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Modified APC-Resistance Test: Variable Ratios With Respect to Source of Factor V-Deficient Plasma

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A single point mutation of the factor V (FV) gene, leading to the substitution Arg506Gln in the FV molecule (FV-Leiden) and hence resistance to its breakdown by activated protein C (APC), is the most prevalent risk factor for venous thrombosis in the Caucasians. A ratio determined by activated partial thromboplastin time (APTT) of test plasma in the presence or absence of exogenous APC (the APC ratio), is the method widely used to screen individuals with this risk factor for thrombosis. Because of functional defects of vitamin K-dependent clotting factors in patients on oral anticoagulant therapy, this method cannot be applied to those patients without modification. One modification is to mix test plasma (1:5 or 1:10) with FV-deficient plasma so that 80–90% of functioning vitamin K-dependent factors are supplied by the FV-deficient plasma. Even with 10–20% of FV in the mixture, APC-resistance still can be demonstrated. In this report, we present our results of the modified APC-sensitivity assay using FV-deficient plasma from different commercial sources. APC ratios determined by the original method in which test plasma is not mixed with FV-deficient plasma can be significantly different from those determined by the modified method in which test plasma is diluted 1:5 with FV-deficient plasma. This difference between methods was observed not only in normal individuals, but also in FV-Leiden positive individuals, and in patients on warfarin therapy. Further, APC ratios varied significantly depending on the commercial source of the FV-deficient plasma. The modified method is apparently suitable to identify APC-resistance in patients on warfarin therapy, as well as in individuals not receiving anticoagulant treatment. However, one must be aware that APC-resistance ratios obtained with the modified method are likely to be different from those established with the original method, and the source of FV-deficient plasma can be a factor influencing the ratios in the former cases. Am. J. Hematol. 54:214–218, 1997 © Wiley-Liss, Inc.

Key words: Modified APC-resistance test; factor V-deficient plasma; warfarin therapy

INTRODUCTION

A single point mutation in the factor V (FV) gene, leading to an Arg506Gln substitution in factor V (FV-Leiden), is the most prevalent risk factor identified to date for venous thrombosis in Caucasians [1–5]. The FV-Leiden mutation results in the resistance of factor V_a to breakdown by activated protein C (APC). It is important for clinical laboratories in Europe and America to be able to screen for this abnormality routinely and accurately. The original APC-resistance screening method described by Dahlback et al. [6] was simple and, with the availability of a commercial assay kit, has been widely adopted. Because of multiple clotting factor deficiency in patients on oral anticoagulant therapy, the clotting time-based original method cannot be used to detect APC-resistance in those patients. Therefore, molecular genetic testing for FV-Leiden is needed. However, the use of a DNA-based assay as a screening test for FV-Leiden may not be justifiable economically and presents technical difficulties for laboratories in small hospitals. Several recent articles have described a modified APC-resistance test in which the test plasma is diluted with FV-deficient plasma in a ratio of 1:5 or 1:10 [7–10]. The modified test can be used for patients on oral anticoagulant therapy and

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is possibly more sensitive than the original assay in which the test plasma is not diluted [6,11].

The advantage of being able to test patients on oral anticoagulant therapy with the modified method outweighs the additional cost incurred by requiring FV-deficient plasma. In the process of replacing the original with the modified method in our laboratory, we noticed discrepant APC ratios derived from these two techniques in asymptomatic healthy individuals. The ratios obtained from the modified method were significantly lower than those from the original method, and our findings were opposite to what has been reported in the literature. One report stated that the ratios derived from the original and modified methods on normal individuals are not statistically different [8], whereas another showed a rise from a ratio of 2.3 with the original method to >3.0 with the modified method [9]. We suspected that the source of FV-deficient plasma might be a contributing factor to the discrepancy and proceeded to examine APC ratios derived from the original and modified techniques, with special attention to the source of FV-deficient plasma, in normal individuals and in asymptomatic individuals with FV-Leiden. Further, we tested patients on warfarin therapy to see whether APC-resistance can be revealed by the modified method. We wish to share our experience with those who are contemplating replacing their original APC-resistance testing with the modified method.

MATERIALS AND METHODS

Normal and Patient Blood Samples

In this study, blood from 27 healthy laboratory and medical personnel was drawn with consent. Fifteen of the donors were female; none of the donors had history of thrombosis. In an earlier study for establishing the reference range of APC-resistance ratio, blood from 45 healthy donor was tested. Again, none of those individuals had history of thrombosis. Ten of the original 45 donors, including four confirmed to have heterozygous FV-Leiden by restriction enzyme assay (see below), donated again for the present study. Seventeen of the donors participating in the present study had not been tested previously for APC-resistance. Blood samples from 40 consecutive patients on warfarin therapy were also tested for APC-resistance. Blood samples from these patients were surplus samples from prothrombin time testing ordered by their physicians.

All blood samples were drawn with vacutainers containing a 3.8% sodium citrate solution with a ratio of 9 parts blood and 1 part citrate solution. Blood was double-spun at 3,000 rpm, 15 min each. Plasma used for this study was transferred to another tube and frozen at −20°C for 2 to 5 days and thawed once.

APC-Resistance Test Kits and FV-Deficient Plasmas

The ''research'' formulation of APC-testing kits, which differs from the ''diagnostic'' formulation by not containing positive and negative control plasmas in the kit, were purchased from Chromogenix (Molndal, Sweden). Positive and negative control plasmas from a ''diagnostic'' kit used for diagnostic purpose were tested with reagents in the ''research'' kit. FV-deficient plasmas were obtained from three sources: Instrumentation Laboratories (IL, Lexington, MA), George King (GK, Overland, KS) and Chromogenix. All claim <1% FV activity in the deficient plasma.

APTT Reagent and Instrumentation

Partial thromboplastin reagent used in this study was provided by Chromogenix and was included in the APC-resistance test kit. An IL ACL-3000 coagulation instrument was used for APTT determinations. APC-resistance ratio was obtained in accordance with what has been described in the literature [6–10]. In the modified APC-resistance test, test plasma was diluted 1:5 with one of the FV-deficient plasmas. The original [6] APC-resistance test was performed on samples of all normal individuals, and on 15 of the 40 samples of patients on coumadin therapy; the modified [7–10] test was applied to all samples.

Genomic Determination of FV-Leiden

Genomic determination of FV-Leiden was performed in the following individuals: In the group of 27 healthy individuals, two with APC-resistance ratio above the reference mean and 6 with the ratio below 2 SD of the mean were tested. In the group of 40 patients on oral anticoagulants, nine with the ratio below the mean and one at the mean (using Chromogenix FV-deficient plasma in the assay) were tested. Genomic DNA was extracted from leukocytes in the buffy coat using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). The extracted DNA served as the template for polymerase chain reaction (PCR) amplification of the region surrounding codon 506 of the factor V gene. An upstream primer, 5′-TGCCCAGTGCTTAACAAGACCA-3′, and a down stream primer, 5′-TGTTATCACACTGGTGCTAA-3′ from Gibco-BRL (Grand Island, NY) were used to amplify the DNA segment. Amplified DNA products were digested with Mnl-1 (New England BioLab, Boston, MA). Both digested and undigested amplification products were loaded onto 3% agarose gel and electrophoresed. The presence of a 20 base pair restriction fragment in Mnl-1 digested products was indicative of factor V-Leiden mutation. Simultaneous presence of a 200 base pair and a 163 base pair product was indicative of heterozygosity for FV-Leiden [7,8,12].

TABLE I. Paired *t*-Test of APTT Results of Normal Individuals (n = 21) Obtained From the Original and Modified Methods in the Absence or Presence of APC*

	Undiluted	IL FV-def.	GK FV-def.	Chrom FV-def
No APC	33.9 ± 2.8	37.8 ± 2.3	41.3 ± 2.9	35.3 ± 1.3
P		<0.001	<0.001	0.101
With APC	131.9 ± 23.1	127.2 ± 13.1	107.4 ± 20.3	87.9 ± 7.6
P		0.303	<0.001	<0.001

*Plasma was tested undiluted (original method) or diluted 1:5 with FV-deficient plasma from three commercial sources. Mean ± SD; *P:* comparing to undiluted. IL: Instrumentation Laboratories; GK: George King; Chrom: Chromogenix; def.: deficient plasma.

Comparison of Data

Actual APTT results of normal individuals obtained with the original method in the absence or presence of APC, and APC-resistance ratios of these individuals derived from APTT results, were compared with those obtained from the modified technique in which the test plasma was diluted 1:5 with FV-deficient plasma from three sources, by paired *t*-test. Significant differences were considered with *P* values <0.05.

RESULTS

APTT of Healthy Individuals Obtained With Original and Modified Methods

In healthy individuals, APTT in seconds obtained with the modified method in the absence of APC were generally longer than those obtained from the original method. In the presence of APC, APTT in seconds were shorter with the modified method than with the original method. The extent of difference in seconds between these two methods was related to the source of FV-deficient plasma. This was particularly evident when APC was present and when the FV-deficient plasma was from Chromogenix. For example, an average APTT of 132 sec obtained with the original method could be reduced to 88 sec with the modified method with FV-deficient plasma from Chromogenix (Table 1).

APC-Resistance Ratios of Healthy Individuals Obtained with Original and Modified Methods

Six of 27 healthy individuals had APC-resistance ratios below 2 SD of the reference mean established earlier. These 6 asymptomatic individuals were heterozygous for FV-Leiden by genomic determination. Irrespective of being normal or harboring the FV-Leiden gene, the mean APC-resistance ratio of normal individuals obtained with the original method (test plasma not diluted) was higher than that obtained with the modified method (test plasma diluted 1:5 with FV-deficient plasma) (Fig. 1). Based on paired *t*-test, the difference in mean APC-resistance ratios obtained with the original and modified methods was statistically significant (Table II). Further, the mean

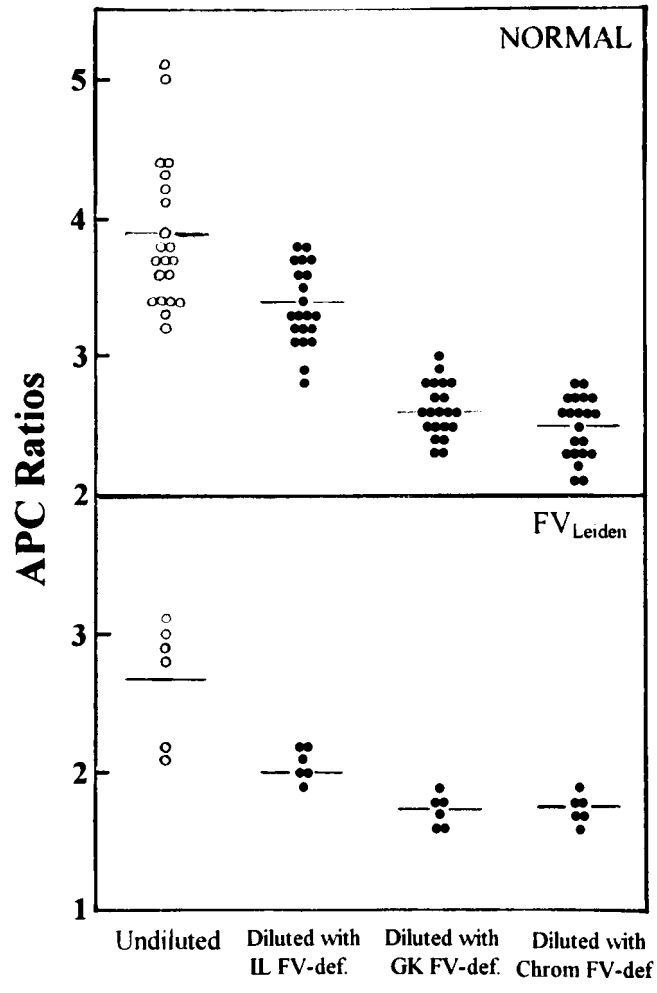

Fig. 1. APC ratios of normal individuals (n = 21) and individuals with confirmed FV-Leiden (n = 6) whose plasma was either tested undiluted or diluted 1:5 with FV-deficient plasma from one of the three commercial sources. Horizontal lines indicate means. The ratio derived from the undiluted method is significantly higher than those from the diluted methods.

APC-resistance ratio obtained from the modified method using FV-deficient plasmas from different sources was significantly different. For example, the ratio obtained with FV-deficient plasma from IL was different from those obtained from George King or Chromgenix (Table II).

TABLE II. Paired *t*-Test of APC Ratios Determined With Original and Modified APC-Resistance Test Methods on Normal Individuals (n = 21) and Individuals With FV_{Leiden} (n = 6)*

	Undiluted	IL FV-def.	GK FV-def.	Chrom FV-def.
Normal	3.9 ± 0.6	3.4 ± 0.3	2.6 ± 0.2	2.5 ± 0.2
P		<0.001	<0.001	<0.001
FV_{Leiden}	2.7 ± 0.4	2.1 ± 0.1	1.7 ± 0.1	1.8 ± 0.1
P		0.009	<0.003	0.003

*Plasma was tested undiluted (original method) or diluted 1:5 with FV-deficient plasma from three sources. Mean ± SD; *P:* comparing to undiluted. IL: Instrumentation Laboratories; GK: George King; Chrom: Chromogenix; def: deficient plasma.

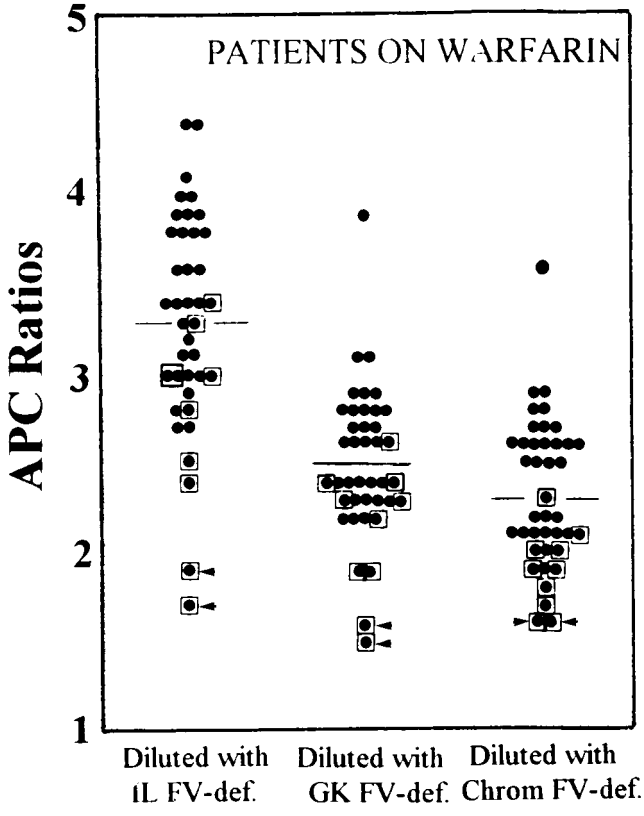

Fig. 2. APC ratios of patients on oral anticoagulant therapy, determined by the modified method in which test plasma was 1:5 diluted with one of the FV-deficient plasmas. Again, depending on the source of the FV-deficient plasma, the ratio can be significantly different with different FV-deficient plasma used. Blocked dots indicate samples that have been assayed for presence of FV-Leiden by a DNA-based method; arrowheads indicate individuals tested FV-Leiden positive.

APC Ratios of Patients on Warfarin Therapy

More than half of patient samples tested with the original method (test plasma not diluted with FV-deficient plasma) did not clot (no clotting by 259 sec) in the presence of APC. Those that clotted had very prolonged

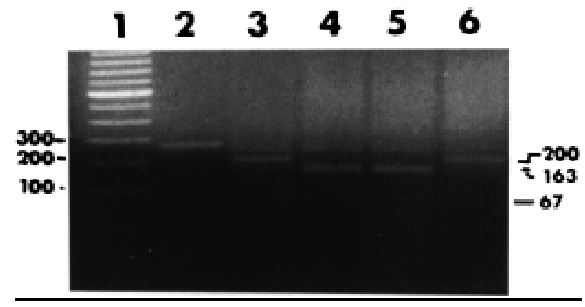

Fig. 3. Restriction enzyme digest products following PCR amplification of the region surrounding factor V codon 506. Lane 1: Molecular weight marker in base pairs. Lane 2: Undigested 267 base pair PCR product. Lane 3: FV-Leiden positive (mutated FV gene) control. Lane 4: FV-Leiden negative (wild type) control. Lane 5: A FV-Leiden negative patient on warfarin therapy whose APC ratios returned by the modified APC-resistance test were 2.4/1.9/1.8 using IL/GK/Chrom FV-deficient plasma as "diluents." Lane 6: A FV-Leiden positive (mutation, heterozygous) patient on warfarin therapy whose APC ratios were 1.6/1.5/1.7 by the modified method using same FV-deficient plasma as shown on lane 5 as "diluents."

APTT readings ranging from 85 to 242 seconds. APC ratios of patients on warfarin therapy obtained with the modified method varied depending on the source of the FV-deficient plasma used in the test (Fig. 2).

Molecular Detection of Factor V-Leiden in Patient Samples

Six of the 27 asymptomatic healthy individuals had APC ratios below 2 SD of the mean. They all were FV-Leiden positive. Two random individuals with APC ratios above the mean were FV-Leiden negative.

Of the nine samples from patients on warfarin therapy whose APC-resistance ratios were below the mean, two were FV-Leiden positive. These two samples had APC ratios 2 or 3 SD below the mean. The other 7 patients whose APC-resistance ratios were <2 SD of the mean and the one patient whose APC-resistance ratio was at the mean were FV-Leiden negative. Mnl-I restriction enzyme analysis of Arg506Gln mutation of two patients, one FV-Leiden negative and one positive, is illustrated in Figure 3.

DISCUSSION

The finding that the APC-resistance test could be performed on plasma from patients on warfarin treatment by a simple dilution of test plasma with FV-deficient plasma was a major application improvement of the original technique for screening APC-resistance individuals [7–10]. In our evaluation of the modified test, we noticed much lower APC ratios obtained with the modified method than with the original method in normal as well

as asymptomatic FV-Leiden positive individuals. These findings were exactly opposite to what has been reported in the literature [8,9]. We suspected the source of FV-deficient plasma could be the main reason for the discrepancy and proceeded to test the hypothesis. With FV-deficient plasma from three commercial sources, the modified method consistently returned lower ratios than did the original method. The decrease in APC ratio was mainly a result of shortened APTT in samples diluted with FV-deficient plasma when APC was present.

The *extent* of APC ratio difference among the methods was related to the source of FV-deficient plasma. Earlier, Kraus and Wagner [13] described instrument- and reagent-dependent variation in APC ratios with the original method.

Because of the small number of individuals with FV-Leiden in our pool, we could not reach a firm conclusion that the modified method is more sensitive and specific than the original one. However, the modified method does have a smaller standard deviation from the mean than the original method. It seems that the modified method is suitable for APC-resistance testing, and is able to identify resistant individuals who are on oral anticoagulant therapy. Our findings do support a recent publication that the modified test can be used in patients without receiving oral anticoagulant treatment [14], provided a new cutoff APC ratio based on the FV-deficient plasma used is established. It should be noted that with FV-deficient plasma from certain sources, detection of heterozygous FV-Leiden might be difficult.

In summary, APC ratios obtained with the modified method vary significantly depending on the source of FV-deficient plasma. Since many laboratories may replace the original method with the modified one, it is important for individual laboratories to carefully select the FV-deficient plasma and establish reference ratios based on the source of FV-deficient plasma.

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